

WHAT IS CLAIMED IS:

5 1. A glycosylated polypeptide comprising the primary structure

NH₂-X-Pp-COOH, wherein:

X is a peptide addition comprising or contributing to a glycosylation site, and
Pp is a polypeptide of interest.

2. A glycosylated polypeptide comprising the primary structure NH₂-P_x-X-P_y-

10 COOH, wherein:

P_x is an N-terminal part of a polypeptide Pp of interest,

P_y is a C-terminal part of said polypeptide Pp, and

X is a peptide addition comprising or contributing to a glycosylation site.

3. The polypeptide of claim 1, wherein Pp is a mature polypeptide.

4. The polypeptide of claim 2, wherein P_x is a non-structural N-terminal part of
a mature polypeptide Pp, and P_y is a structural C-terminal part of said mature polypeptide.

5. The polypeptide of claim 1, wherein Pp is a native polypeptide.

6. The polypeptide of claim 1, wherein Pp is a variant of a native polypeptide.

7. The polypeptide of claim 6, wherein Pp comprises at least one introduced
and/or at least one removed glycosylation site for a non-peptide moiety as compared to the
corresponding native polypeptide.

8. The polypeptide of claim 1, wherein Pp is of mammalian origin.

9. The polypeptide of claim 8, wherein Pp is of human origin.

10. The polypeptide of claim 1, wherein Pp is a therapeutic polypeptide.

25 11. The polypeptide of claim 1, wherein Pp is selected from the group consisting
of an antibody or antibody fragment, a plasma protein, an erythrocyte or thrombocyte protein, a
cytokine, a growth factor, a profibrinolytic protein, a protease inhibitor, an antigen, an enzyme, a
ligand, a receptor, or a hormone.

5

12. The polypeptide of claim 1, wherein Pp is of microbial origin.

13. The polypeptide of claim 12, wherein Pp is a microbial enzyme.

14. The polypeptide of claim 13, wherein Pp is selected from the group consisting of protease, amylase, amyloglucosidase, pectinase, lipase and cutinase.

15. The polypeptide of claim 1, wherein X comprises 1-500 amino acid residues.

10

16. The polypeptide of claim 15, wherein X comprises 2-50 amino acid residues, such as 3-20 amino acid residues.

17. The polypeptide of claim 1, wherein X comprises 1-20, in particular 1-10 glycosylation sites.

18. The polypeptide of claim 1, wherein X comprises at least one glycosylation site within a stretch of 30 amino acid residues, such as at least one within 20 amino acid residues, in particular at least one within 10 amino acid residues, in particular 1-3 glycosylation sites.

19. The polypeptide of claim 1, wherein X comprises at least two glycosylation sites, wherein two of said sites are separated by at most 10 amino acid residues, none of which comprises a glycosylation site.

20. The polypeptide of claim 6, wherein the polypeptide Pp is a variant of a native polypeptide which, as compared to said native polypeptide, comprises at least one introduced or at least one removed glycosylation site.

21. The polypeptide of claim 20, wherein the polypeptide Pp comprises at least one introduced glycosylation site, in particular 1-5 introduced glycosylation sites.

25

22. The polypeptide of claim 1, wherein X has an N residue in position -2 or -1, and Pp has a T or an S residue in position +1 or +2, respectively, the residue numbering being made relative to the N-terminal amino acid residue of Pp.

23. The polypeptide of claim 1, wherein X has the structure X₁-N-X₂-[T/S]-Z, wherein X₁ is a peptide comprising at least one amino acid residue or is absent, X₂ is any amino

5 acid residue different from a proline residue, and Z is absent or a peptide comprising at least one amino acid residue, the N-terminal amino acid residue of which is different from a proline.

24. The polypeptide of claim 23, wherein X₁ is absent, X₂ is an amino acid residue selected from the group consisting of I, A, G, V and S, and Z comprises at least one amino acid residue, the N-terminal amino acid residue of which is different from proline.

10 25. The polypeptide of claim 24, wherein Z is a peptide comprising 1-50 amino acid residues, preferably comprising 1-10 glycosylation sites.

26. The polypeptide of claim 25, wherein X₁ comprises at least one amino acid residue, X₂ is an amino acid residue selected from the group consisting of I, A, G, V and S, and Z is absent.

27. The polypeptide of claim 26, wherein X₁ is a peptide comprising 1-50 amino acid residues, preferably comprising 1-10 glycosylation sites.

28. The polypeptide of claim 1, wherein X comprises a peptide sequence selected from the group consisting of INA[T/S], GNI[T/S], VNI[T/S], SNI[T/S], ASNI[T/S], NI[T/S], SPINA[T/S], ASPINA[T/S], ANI[T/S]ANI[T/S]ANI, ANI[T/S]GSNI[T/S]GSNI[T/S], FNI[T/S]VNI[T/S]V, YNI[T/S]VNI[T/S]V, AFNI[T/S]VNI[T/S]V, AYNI[T/S]VNI[T/S]V, APND[T/S]VNI[T/S]V, ANI[T/S], ASNS[T/S]NNG[T/S]LNA[T/S], ANH[T/S]NE[T/S]NA[T/S], GSPINA[T/S], ASPINA[T/S]SPINA[T/S], ANN[T/S]NY[T/S]NW[T/S], ATNI[T/S]LNY[T/S]AN[T/S]T, AANS[T/S]GNI[T/S]ING[T/S], AVNW[T/S]SND[T/S]SNS[T/S], GNA[T/S], AVNW[T/S]SND[T/S]SNS[T/S], ANN[T/S]NY[T/S]NS[T/S], ANNTNYTNWT, ANI[T/S]VNI[T/S]V, ND[T/S]VNF[T/S] and NI[T/S]VNI[T/S]V wherein [T/S] is either a T or an S residue, preferably a T residue.

29. The polypeptide of claim 1, wherein the peptide addition X comprises the sequence NSTQNATA or ANLTVRNLTRNVTV.

30. The polypeptide of claim 1, further comprising an attachment group for a second non-peptide moiety, said attachment group being linked to the second non-peptide moiety.

5 31. The polypeptide of claim 30, wherein the non-peptide moiety is selected
from the group consisting of a polymer molecule, a lipophilic group and an organic derivatizing
agent.

10 32. The polypeptide of claim 30, wherein the attachment group for the non-
peptide moiety is one present on an amino acid residue selected from the group consisting of the
N-terminal amino acid residue, the C-terminal amino acid residue, lysine, cysteine, arginine,
glutamine, aspartic acid, glutamic acid, serine, tyrosine, histidine, phenylalanine and tryptophan.

15 33. The polypeptide of claim 30, wherein the polypeptide Pp is a variant of a
native polypeptide, which as compared to said native polypeptide, comprises at least one
introduced and/or at least one removed attachment group for the second non-peptide moiety.

20 34. The polypeptide of claim 33, wherein the polypeptide Pp comprises at least
one introduced attachment group, in particular 1-5 introduced attachment groups.

25 35. The polypeptide of claim 1, which has a molecular weight of at least 67 kDa,
in particular at least 70 kDa.

30 36. The polypeptide of claim 1, which has at least one of the following
properties relative to the polypeptide Pp, the properties being measured under comparable
conditions:

35 *in vitro* bioactivity which is at least 25% of that of the polypeptide Pp as
measured under comparable conditions, increased affinity for a mannose receptor or other
carbohydrate receptors, increased serum half-life, increased functional *in vivo* half-life, reduced
renal clearance, reduced immunogenicity, increased resistance to proteolytic cleavage, improved
targeting to lysosomes, macrophages and/or other subpopulations of human cells, improved
stability in production, improved shelf life, improved formulation, improved liquid formulation,
improved purification, improved solubility, and improved expression.

40 37. A nucleotide sequence encoding the polypeptide according to claim 1.

45 38. A vector comprising the nucleotide sequence according to claim 37.

5 39. A host cell transformed or transfected with the nucleotide sequence of claim
37, or a vector according to claim 38.

40. The host cell of claim 39, which is a glycosylating host cell.

41. The host cell of claim 40, which is a mammalian cell, an invertebrate cell such as an insect cell, a yeast cell or a plant cell, or a transgenic animal.

10 42. A method of producing the polypeptide of claim 1, comprising culturing a host cell according to claim 39 under conditions permitting expression of the polypeptide and recovering the polypeptide from the culture.

15 43. A method of producing a polypeptide according to claim 30, attached to a second non-peptide moiety, which method comprises subjected the polypeptide to conjugation to the non-peptide moiety under conditions for the conjugation to take place.

44. The method of claim 43, wherein the polypeptide is prepared by the method of claim 42.

20 45. A method of preparing a nucleotide sequence according to claim 37, which method comprises

a) subjecting a nucleotide sequence encoding the polypeptide Pp to elongation mutagenesis;

b) expressing the mutated nucleotide sequence obtained in step a) in a suitable host cell;

c) optionally conjugating polypeptides expressed in step b) to a second non-peptide moiety;

25 d) selecting polypeptides obtained in step b) or c) which comprises at least one oligosaccharide moiety and optionally a second non-peptide moiety attached to the peptide addition part of the polypeptide; and,

e) isolating a nucleotide sequence encoding the polypeptide selected in step d).

5 46. The method of claim 45, which further comprises screening polypeptides resulting from step b) or c) for at least one improved property, and wherein the selection step d) further comprises selecting polypeptides having such improved property.

47. The method of claim 45, wherein the elongation mutagenesis is conducted so as to enrich for codons encoding an amino acid residue comprising a glycosylation site.

10 48. The method of claim 45, wherein the elongation mutagenesis is conducted so as to enrich for codons required for introduction of an attachment group for a second non-peptide moiety.

15 49. The method of claim 44, which further comprises subjecting the part of the nucleotide sequence encoding Pp to mutagenesis to remove and/or introduce glycosylation sites and optionally amino acid residues comprising an attachment group for the second non-peptide moiety.

50. The method of claim 45, wherein the selection in step d) is performed so as to select a conjugate having at least one of the properties defined in claim 36.

20 51. A method of producing a glycosylated polypeptide encoded by a nucleotide sequence prepared according to the method of claim 45, wherein the nucleotide sequence encoding the polypeptide selected in step c) is expressed in a glycosylating host cell and the resulting glycosylated expressed polypeptide is recovered.

25 52✓ A method of improving one or more selected properties of a polypeptide Pp of interest, which method comprises

a) preparing a nucleotide sequence encoding a polypeptide with the primary structure:

NH₂-X-Pp-COOH,

wherein

X is a peptide addition comprising or contributing to a glycosylation site that is capable of conferring the selected improved property/ies to the polypeptide Pp;

b) expressing the nucleotide sequence of a) in a suitable host cell;

5 c) optionally conjugating the expressed polypeptide of b) to a second non-peptide moiety; and,
 d) recovering the polypeptide resulting from step c).

53. The method of claim 52, wherein the polypeptide Pp and/or the peptide addition X is as defined in claim 1.

10 54. The method of claim 52, wherein the nucleotide sequence of step a) is prepared by subjecting a nucleotide sequence encoding the polypeptide Pp to random elongation mutagenesis.

15 55. The method of claim 54, wherein the random elongation mutagenesis is conducted so as to enrich for codons encoding an amino acid residue comprising or contributing to a glycosylation site and/or an attachment group for the second non-peptide moiety.

20 56. The method of claim 52, wherein, in the preparation of the nucleotide sequence of a), the part of the nucleotide sequence encoding the polypeptide Pp is subjected to mutagenesis to remove and/or introduce a glycosylation site or to remove and/or introduce an attachment group for a second non-peptide moiety.

25 57. The method of claim 52, wherein the property/ies to be improved is/are selected from the properties defined in claim 37.

PCT/US2003/035000